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Coarse and Fine Control and Annual Changes of Sucrose-Phosphate Synthase in Norway Spruce Needles[1]

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Annual changes of activity of sucrose-phosphate synthase (SPS) from spruce (*Picea abies* [L.] Karst.) needles were studied with respect to three regulatory levels: metabolic fine control, covalent modification (phosphorylation), and protein amount. Glucose-6-phosphate served as an allosteric activator of spruce SPS by shifting the Michaelis constant for the substrate fructose-6-phosphate from 4.2 to 0.59 mM, whereas inorganic phosphate competitively inhibited this activation. The affinity for the other substrate, UDP-glucose, was unaffected. Incubation of the crude extract with ATP resulted in a time- and concentration-dependent decrease of the maximal velocity of SPS. This inactivation was sensitive to staurosporine, a potent protein kinase inhibitor, indicating the participation of a protein kinase. Probing SPS protein with heterologous antibodies showed that the subunit of spruce SPS is an approximately 139-kD protein and that changes in the extractable activity during the course of a year were correlated with the amount of SPS protein. High SPS activities in winter were paralleled by increased levels of the activator glucose-6-phosphate and the substrate fructose-6-phosphate, indicating a high capacity for sucrose synthesis that may be necessary to maintain photosynthetic CO_2 fixation in cold-hardened spruce needles.

The control of Suc synthesis constitutes an important factor in the regulation of Suc export, photosynthesis, and starch formation. One of the key enzymes in the control of Suc synthesis is SPS, and its activity can be regulated via metabolic fine control, coarse control by covalent modification, and by the amount of enzyme protein. Metabolic fine control is exerted by the allosteric effectors Glc-6-P and Pi. Glc-6-P, an intermediate of Suc synthesis, activates, whereas Pi inhibits SPS activity in most tissues (Doehlert and Huber, 1983; Huber et al., 1989b; Reimholz et al., 1994). Some species exhibit a light-dependent covalent modification of SPS. Accordingly, the enzymes can be classified into three different types (for review, see Huber and Huber, 1992). Organisms such as maize and other monocotyledons show a light-induced increase in V_{max} under nonlimiting substrate conditions. In other plants (e.g. spinach), V_{max} is not influenced by a dark → light transition; in contrast, an increase in SPS activity in the presence of limiting substrate concentrations can be observed. In several species such as soybean, SPS activity is not affected by dark → light transitions. Covalent modification is due to phosphorylation/dephosphorylation. SPS from spinach, for instance, can be inactivated by phosphorylation of specific Ser residues by an ATP-dependent SPS kinase. This effect is reversed by a protein phosphatase 2A, which dephosphorylates the enzyme. The dephosphorylated protein has a higher affinity for its activator Glc-6-P, whereas the affinity for the inhibitor Pi is lowered. Thus, covalent modification is connected with allosteric regulation. Finally, a correlation of the SPS protein level with the SPS activity has been observed in developing (Huber and Huber, 1992) and cold-treated (Guy et al., 1992) spinach leaves.

Most of the results described above were obtained by investigating leaf SPS of annual crops, whereas little is known about the enzyme in leaves of evergreen conifers. Until now, to our knowledge, nothing has been published about the regulatory properties of spruce (*Picea abies* [L.] Karst.) needle SPS. Recent investigations have shown that the extractable activity of SPS in spruce needles undergoes characteristic annual changes, with the highest activities occurring in winter (October to February) and decreasing activities occurring in summer (Egger and Hampp, 1993; Egger et al., 1996). The variations in activity were understood to be an adaptation to low temperatures in winter and to the demand for Suc export, which is highest in early spring. The objective of our work, therefore, was to study the regulation of Norway spruce needle SPS in more detail. Our data show that spruce SPS activity is modulated by allosteric effectors as well as by ATP. Annual changes in activity are, however, mainly due to the changing amount of enzyme protein.

MATERIALS AND METHODS

Mature needles from the same shoots (9 months old at the beginning of the sampling period) were collected continuously from 20-year-old Norway spruce (*Picea abies* [L.] Karst.) trees in the field (Edelmannshof, Welzheimer Wald, Germany) from February 1990 through February 1991 between 11 AM and 1 PM. Samples were frozen immediately in liquid nitrogen and stored at −80°C. Homogenization and lyophilization of the samples were performed as de-

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Abbreviations: PGI, phosphoglucose isomerase; SPS, Suc-P synthase; $v_{+Glc\text{-}6\text{-}P}$, velocity in the presence of 10 mM Glc-6-P; $v_{-Glc\text{-}6\text{-}P}$, velocity in the absence of Glc-6-P; V_{max}, maximal velocity.

scribed by Einig and Hampp (1990). The dried plant powder was kept under vacuum at −30°C until use.

Extraction and Determination of Enzyme Activities and Metabolites

Enzyme activities were measured according to the method of Egger and Hampp (1993) by applying microplate reader assays. The decrease or increase of NADH was recorded on-line using a microplate reader (340 ATTC; Salzburger Labortechnik, Salzburg, Austria) in 96-well microtiter plates (Costar, Cambridge, MA). The mean values of two independent assays, four parallel determinations each, were evaluated.

Native extracts were obtained by incubating 4 mg of freeze-dried material with 1 mL of ice-cold extraction buffer containing 5% (w/v) PVP (M_r 10,000), 100 mM/300 mM Tris/borate buffer, pH 7.6, 1 mM β-mercaptoethanol, and 0.85% (w/v) BSA. Before centrifugation (12,000*g*, 8 min, 4°C), the suspension was kept on ice for 5 min and mixed occasionally (Egger and Hampp, 1993). The cooled supernatants were used immediately for measuring enzyme activities.

The SPS assay contained 1.13 units of lactate dehydrogenase, 0.72 units of pyruvate kinase, 50 mM Mops/NaOH, pH 7.5, 10 mM Fru-6-P, 12 mM Glc-6-P, 15 mM $MgCl_2$, 0.8 mM PEP, 0.3 mM NADH, 5 mM UDP-Glc, and 10 μL of native extract in a final volume of 100 μL per well. To study allosteric regulation of SPS, the concentrations of the substrates Fru-6-P and UDP-Glc as well as the probable allosteric effectors Glc-6-P and Pi were varied.

For the determination of activity of PGI, 10 μL of native extract (about 0.9 μg of protein) was added to 80 μL of reagent, resulting in a final assay concentration of 0.25 unit of Glc-6-P dehydrogenase from *Leuconostoc mesenteroides*, 50 mM Mops/NaOH, pH 7.5, 1 mM NAD, and 15 mM $MgCl_2$. The reaction was started by the addition of 10 μL of Fru-6-P (final concentration, 10 mM).

Fru-6-P and Glc-6-P were assayed enzymatically with four parallel measurements each. The resulting NADH was determined after enzymatic cycling in a chromogenic assay mixture (Egger et al., 1996).

ATP-Dependent Inhibition of SPS

ATP- and time-dependent inhibitions of SPS activity were determined by incubating native crude extracts of winter needle samples supplemented with $MgCl_2$ (10 mM) and different ATP concentrations at 25°C. At regular intervals, aliquots were removed and assayed for SPS activity. Participation of a kinase was tested by preincubating crude extracts with 600 nM staurosporine (Sigma) before ATP incubation and determination of SPS activity.

Partial Enzyme Purification, SDS-PAGE, and Western Blotting

SPS from native spruce needle extracts supplemented with 20 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 2 mM DTT, 1 mM PMSF, 4 mM ε-aminocapronic acid, and 4 mM benzamidine, according to Sonnewald et al. (1993), was partially purified over an Ω-aminohexylagarose column. The enzyme was eluted by increasing NaCl concentrations (0–400 mM) in a buffer in which Tris/borate was substituted by 10 mM Hepes, pH 7.4. The SPS activity in each fraction was determined immediately after elution.

For immunological detection, the proteins were precipitated with 80% acetone, resolubilized with Laemmli (1970) buffer, and finally analyzed by SDS-PAGE and western blotting. Electrophoretical separation of proteins was performed in a 7.5% polyacrylamide gel by using the microgel system described by Poehling and Neuhoff (1980). Denaturating extraction for electrophoresis was carried out by mixing 10 mg of dried needle powder and 100 mg of Polyclar AT (Serva, Heidelberg, Germany) with 1 mL of boiling Laemmli (1970) buffer, followed by heating (95°C, 10 min) and centrifugation (12,000*g*, 8 min, 4°C). The supernatants were kept at −20°C until use. Immunological detection of SPS protein was achieved using a semidry western blotting procedure (Hoffmann and Hampp, 1994). The blots were developed with a 1:100 dilution of the antiserum raised against spinach leaf SPS. Final detection was achieved with an alkaline phosphatase kit (Bio-Rad). Calculation of the molecular mass of the immunologically detected proteins was carried out by comparing the migration of the sample and the marker bands using a gel scanner (Elscript 400; Hirschmann, Neuried, Germany). Densitometry reading and subsequent analyses were performed by using the video system Herolab E.A.S.Y. RH (Herolab, Wiesloch, Germany) and National Institutes of Health Image software (version 1.60; http://rsb.info.nih.gov/nih/image-gel; Bethesda, MD).

Protein Determination

The protein content was determined by applying the dot procedure of Neuhoff et al. (1979) with modifications described by Guttenberger et al. (1991).

RESULTS

Metabolic Fine Control of SPS

Because of the lability of the SPS enzyme during purification, crude extracts were used for measurements of enzyme kinetics. Because the regulatory properties of SPS were the same in desalted and crude extracts, further desalting was omitted. The extracts did not contain measurable activities of UDPase and PEP-phosphatase (data not shown), which would have interfered with the assay. Borate, an essential ingredient of the extraction buffer (Guttenberger et al., 1994), inhibited SPS activity by only 10% at 30 mM compared with 1 mM assay concentration (the latter was achieved by concentration and subsequent dilution of the crude extract). Because this inhibition showed no interaction with the allosteric effector Pi, it could be ignored (data not shown).

To avoid the conversion of the substrate Fru-6-P into the allosteric activator Glc-6-P by PGI, the activity of this enzyme was inhibited (>90%) by the addition of 36 mM 6-phosphogluconate (Schnackerz and Noltmann, 1971) without affecting the SPS reaction (data not shown). The

remaining activity of PGI was low enough so that Glc-6-P did not exceed an assay concentration of 0.05 mM, which was neglected.

Effective concentration ranges corresponding to different kinetic reaction orders were defined by measuring SPS activity in the presence of increasing substrate concentrations (0.05–10 mM Fru-6-P or UDP-Glc; data not shown). Concentrations of 0.5, 2, and 5 mM Fru-6-P or 0.5, 1.5, and 5 mM UDP-Glc were chosen to test activation by Glc-6-P and inhibition by Pi, respectively, at different concentration ranges of the substrate saturation curve.

Up to 15 mM Pi did not inhibit SPS activity in the absence of Glc-6-P, as measured under nonsaturating substrate concentrations of Fru-6-P or UDP-Glc (data not shown). In contrast, Glc-6-P caused an increase of SPS activity for all tested substrate conditions (Fig. 1A, Fru-6-P; Fig. 1B, UDP-Glc). This was reflected by a selective change in K_m (Table I). Addition of 10 mM Glc-6-P shifted the K_m for Fru-6-P from approximately 4.2 to 0.59 mM, whereas that for UDP-Glc was not altered. The absolute increase in velocity (the difference in velocity in the presence $[v_{+\text{Glc-6-P}}]$ and in the absence $[v_{-\text{Glc-6-P}}]$ of 10 μM Glc-6-P) was higher at

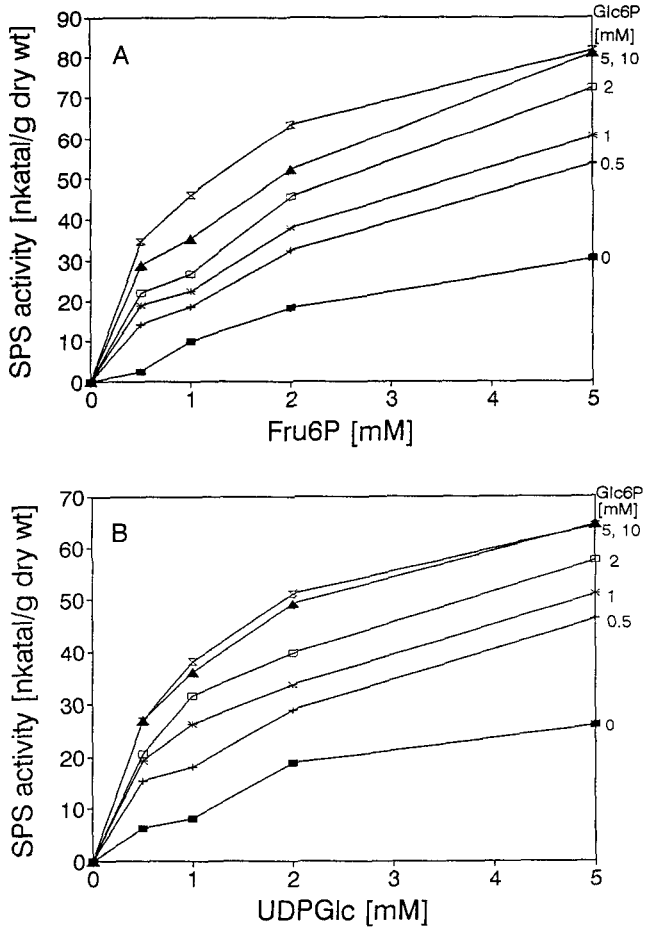

Figure 1. Substrate saturation kinetics for Fru-6-P and UDP-Glc of spruce needle SPS (Glc-6-P-activated). SPS activity was determined in a Fru-6-P-limited (A; 0.5–5 mM Fru-6-P; 5 mM UDP-Glc) and a UDP-Glc-limited (B; 0.5–5 mM UDP-Glc; 10 mM Fru-6-P) assay.

Table I. *K_m of SPS for Fru-6-P and UDP-Glc in the presence of different concentrations of the allosteric activator Glc-6-P*

Glc-6-P	K_m (Fru-6-P)	K_m (UDP-Glc)
mM	mM	mM
0	4.17	1.92
0.5	2.33	1.64
1	1.54	1.64
2	1.60	1.82
5	0.70	1.50
10	0.59	1.56

elevated substrate concentrations (Table II). The relative activation $(v_{+\text{Glc-6-P}}/v_{-\text{Glc-6-P}})$ was, however, higher at lower concentrations of Fru-6-P (Table II). The activation of SPS by Glc-6-P was antagonized by Pi in the Fru-6-P-limited assay (2 mM Fru-6-P, Fig. 2A). This effect was not found for the UDP-Glc-limited assay (Fig. 2B).

Covalent Modification of SPS

When ATP was added to the extraction buffer, SPS activity decreased in a time- and ATP-dependent manner (Figs. 3 and 4). Half-maximal inhibition was achieved with 30 μM ATP. This effect of ATP was obviously due to a kinase reaction, because the decrease in SPS activity was almost completely inhibited by staurosporine (Fig. 5), an inhibitor of many protein kinases (Rüegg and Burgess, 1989). Incubation of crude extracts with ATP, however, did not result in a change in the allosteric behavior of SPS (data not shown). The K_m values for both Fru-6-P and UDP-Glc, as well as the relative activation by Glc-6-P of the ATP-treated enzyme, were in the same range as in the absence of ATP. Thus, only V_{max} was affected. The addition of okadaic acid, an inhibitor of phosphatases 1 and 2A (Huber et al., 1991), to the extraction buffer had no influence on the activity of SPS, independent of ATP.

Partial Purification of SPS and Immunolabeling

Proteins from crude extracts were separated over an Ω-aminohexylagarose column. Two independent purification procedures resulted in fractions with an up to 42-fold increase in specific SPS activity (data not shown).

Cross-reaction of the primary antibody with spruce SPS was tested by western blotting analyses of leaf protein fractions of one experiment with high and low specific SPS activity. Figure 6 shows one dominant immunological cross-reaction in the higher molecular mass range (approximately 139 kD) and three bands in the low molecular mass range (30–50 kD) in the fractions with high specific SPS activity. No cross-reaction was found in samples with low specific SPS activity. Further evidence for the specificity of the antibody was achieved by immunotitration, leading to a 50% decrease in SPS activity of the native extract, when diluted 1:6 with the antiserum (data not shown).

Annual Changes in SPS Activity

SPS in spruce needles exhibits seasonal variation in extractable activity (Egger and Hampp, 1993; Egger et al.,

Table II. *Comparison of the absolute Glc-6-P-mediated increase in velocity as well as the relative activation $v_{+Glc\text{-}6\text{-}P}/v_{-Glc\text{-}6\text{-}P}$ by 10 mM Glc-6-P*

The rate of SPS activity was determined in the absence and presence of 10 mM Glc-6-P at different limiting Fru-6-P concentrations (5 mM UDP-Glc each).

Fru-6-P	$v_{-Glc\text{-}6\text{-}P}$	$v_{+Glc\text{-}6\text{-}P}$	$v_{+Glc\text{-}6\text{-}P} - v_{-Glc\text{-}6\text{-}P}$	$v_{+Glc\text{-}6\text{-}P}/v_{-Glc\text{-}6\text{-}P}$
mM	*nkatal/g dry wt*	*nkatal/g dry wt*	*nkatal/g dry wt*	
0.5	2.4	34.7	32.3	14.6
1	9.8	46.0	36.2	4.7
2	18.3	63.2	45.0	3.5
5	30.4	81.8	51.4	2.7

1996). Thus, we wanted to know whether this is due to changes in the enzyme's regulatory properties or to the amount of enzyme protein.

Determination of needle contents of Fru-6-P and Glc-6-P of one tree showed variations during the course of a year; in general, more Glc-6-P than Fru-6-P was present. Glc-6-P was highest in February of the first year, followed by a continuous decline during spring and summer, reaching the lowest concentrations in August and October, and increasing in February of the following year (Fig. 7). Fru-6-P was low in February of the first year through summer and increased in March and toward autumn/winter (Fig. 7). Variations in the absolute metabolite contents of samples of the same season are referred to individual conditions in the habitat.

The same samples used for the determination of hexose-Ps were taken to assay the seasonal activation state of spruce needle SPS in the presence of different Glc-6-P concentrations (0–10 mM). The decrease of K_m for Fru-6-P in response to Glc-6-P was similar in all samples (Table III), which indicates no substantial season-dependent differences in the allosteric properties of SPS from mature needles. Furthermore, incubation of these samples with 5 mM ATP for 10 min resulted in a decrease in SPS activity of approximately 35%, independent of sampling date (data not shown). According to these findings, covalent modification of SPS by phosphorylation/dephosphorylation does not seem to be involved in the annual regulation of SPS activity.

A direct correlation between SPS activity and SPS protein as determined by western blotting and densitometry reading was verified for three individual trees (Fig. 8), from which one example is shown in detail (Fig. 9). February samples (Fig. 9, lane 2) exhibited both highest activity and amount of SPS protein; however, both of these parameters declined in parallel from March to August (lanes 3–5), indicating that seasonal variation in SPS from mature

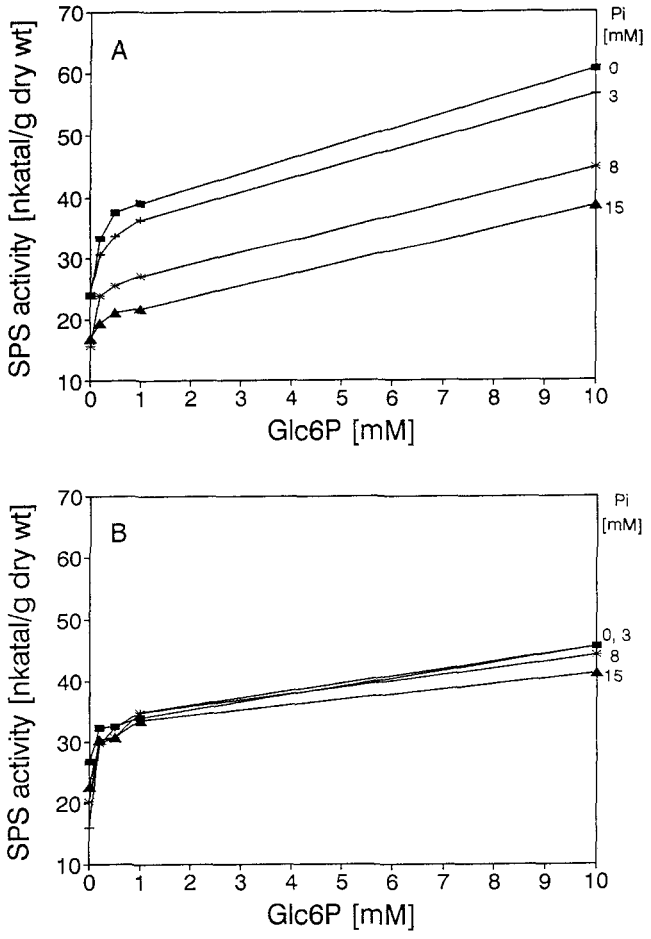

Figure 2. Inhibition of Glc-6-P-activated spruce needle SPS by Pi. SPS activity was measured in a Fru-6-P-limited (A, 2 mM Fru-6-P; 5 mM UDP-Glc) and a UDP-Glc-limited (B, 1.5 mM UDP-Glc; 10 mM Fru-6-P) assay at increasing Glc-6-P concentrations (0–10 mM).

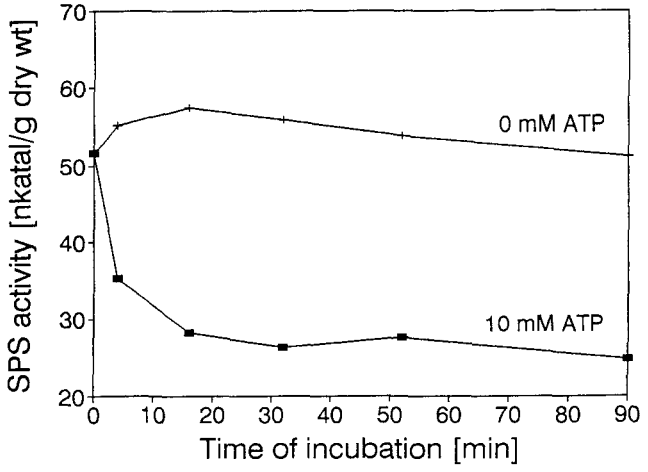

Figure 3. Time-dependent inactivation of spruce needle SPS in the presence (10 mM) or absence of ATP. Crude extracts were incubated at 25°C with the indicated ATP concentrations; at time intervals (0–90 min), aliquots were taken and assayed for V_{max} SPS activity.

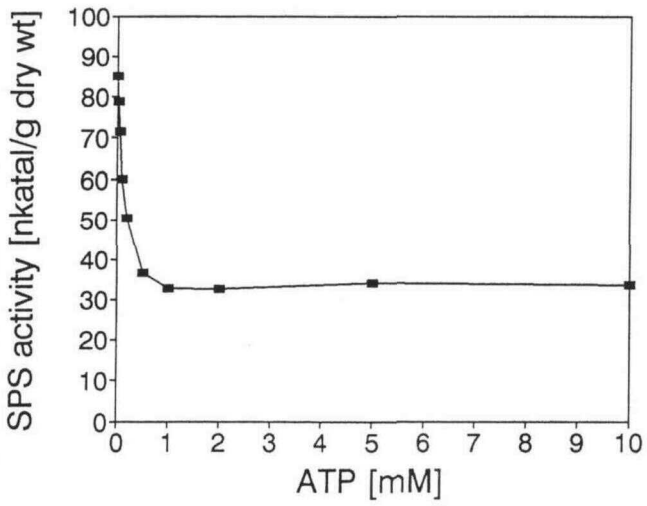

Figure 4. ATP-dependent inactivation of spruce needle SPS. After incubation of crude extracts with 0 to 10 mM ATP (25°C for 10 min), aliquots were taken and assayed for V_{max} SPS activity.

spruce needles occurs by protein level rather than by post-translational modification.

DISCUSSION

SPS is highly regulated (Huber and Huber, 1992), and this regulation can occur at three levels and can vary among different plant species and metabolic situations.

Metabolic Fine Control

SPS activity can be regulated by Glc-6-P and Pi in an allosteric manner. In spruce needles, Glc-6-P activates SPS with a K_a of 0.88 mM (at 0.5 mM Fru-6-P) by decreasing SPS affinity for the substrate Fru-6-P. This results in a K_m shift from 4.2 to 0.59 mM. The activation can be inhibited by Pi

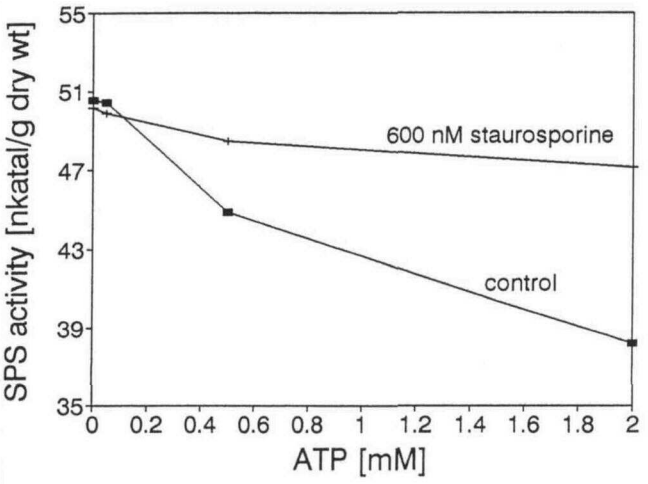

Figure 5. Inhibition of ATP-dependent inactivation of spruce needle SPS by staurosporine. Crude extracts were preincubated in the presence (600 nM) or absence (control) of staurosporine (25°C for 30 min). After addition of up to 2 mM ATP and further incubation for 10 min, aliquots were removed and assayed for V_{max} SPS activity.

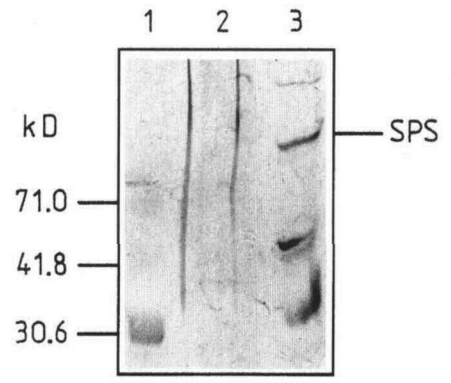

Figure 6. Immunological detection of partially purified spruce needle SPS with a 1:100 dilution of the antiserum, raised against spinach leaf SPS. Lane 1, Protein molecular mass standards; lane 2, eluates with low specific SPS activity (1.25 μg of protein); lane 3, eluates with high specific SPS activity (1.12 μg of protein).

when the concentration of Fru-6-P is rate limiting for SPS. The allosteric regulation measured in vitro seems to be of physiological relevance, as shown by calculations of cytosolic concentrations of Fru-6-P and Glc-6-P, based on data given in Figure 7. Mature spruce needles contain 40 to 140 pmol Fru-6-P mg^{-1} dry weight. This can be converted into 40 to 140 pmol Fru-6-P μL^{-1} cell sap or 0.04 to 0.14 mM Fru-6-P (1 mg dry weight corresponds to 1 μL cell sap; Egger et al., 1996). Since the vacuole constitutes 80% of the needle mesophyll cell volume (Schmidt et al., 1989) and contains no Fru-6-P (Winter et al., 1994), there should be an average of 0.2 to 0.7 mM Fru-6-P in the cytosol and other cell compartments except the vacuole. Because hexose-Ps are predominantly located in the cytosol (Gerhardt et al., 1987), the cytosolic Fru-6-P concentration of spruce needles should exceed 0.2 to 0.7 mM. Similar calculations for Glc-

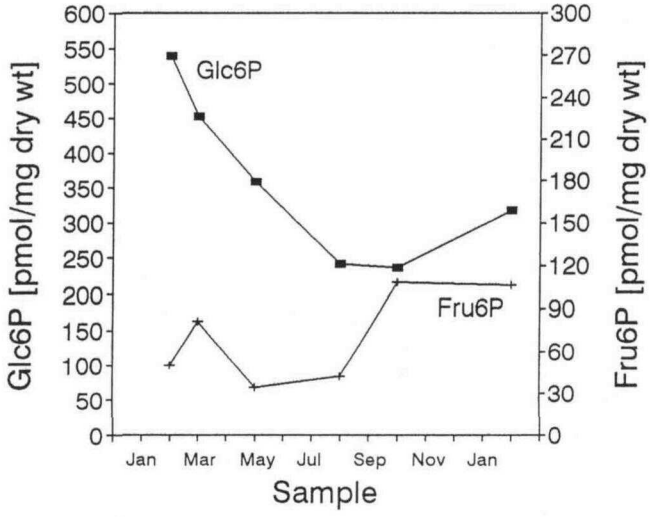

Figure 7. Fru-6-P and Glc-6-P contents of mature spruce needles of the same shoot (9 months old in the beginning of the sampling period) were analyzed at different times of the year. To rule out diurnal changes of hexose-P contents (Einig et al., 1990), each sample was taken at the same time of day.

Table III. *Comparison of allosteric regulation (shifts of K_m for Fru-6-P in the presence of Glc-6-P) of mature needle SPS at different times of the year*

K_m for Fru-6-P was calculated from SPS activities measured at 0.5 to 5 mM Fru-6-P and 5 mM UDP-Glc in the presence of 0 to 10 mM Glc-6-P.

Glc-6-P	Month of Sample Collection				
	February	March	May	August	February
mM			K_m (Fru-6-P) (mM)		
0	4.00	5.21	4.31	2.72	4.17
0.5	1.66	1.80	1.96	1.95	2.33
1	1.28	1.31	1.33	1.30	1.54
2	1.03	1.19	0.81	1.26	1.60
5	0.76	1.13	0.58	0.90	0.70
10	0.59	0.84	0.57	0.64	0.59

6-P result in cytosolic concentrations of least 1.2 to 2.7 mM in Norway spruce leaf cells.

In comparison, Winter et al. (1994) calculated cytosolic concentrations of 2 mM Fru-6-P, 5.9 mM Glc-6-P, and 1.7 mM UDP-Glc for spinach, which are in a range similar to that calculated for spruce needles. These data indicate that the range of K_m values found for spruce needle SPS in crude extracts is of physiological relevance (K_m for Fru-6-P, 4.2–0.59 mM; K_m for UDP-Glc, 1.92–1.56 mM).

Because SPS from partially purified fractions was unstable, the kinetic measurements were carried out with crude extracts of winter samples (needles collected in February). We want to state that our results still impart an objective description because these crude extracts contained only minor amounts of plant-originating compounds; only 4 mg of freeze-dried plant material was added to 1 mL of extraction buffer. This is equivalent to an almost desalted protein solution: measurements of HCl-soluble Pi in mature spruce needles, for example, result in values of less than 10 nmol Pi mg^{-1} dry weight (Egger et al., 1996); consequently, extracts of 4 mg plant material mL^{-1} contain about 40 μM Pi and even much less Glc-6-P. An interaction of secondary metabolites can be neglected because further desalting showed no changes in the regulatory properties of SPS. Because the enzyme exhibited a more or less constant activity for 90 min when incubated at 25°C in the crude extract (see Fig. 3, "0 mM ATP"), proteases do not interfere significantly. In addition, under the present assay conditions, a linear kinetic of SPS activity can be observed for more than 1 h (see also Egger and Hampp, 1993). Moreover, some possibly disturbing factors were ruled out: the inhibition of SPS activity by borate showed no interaction with the phosphate effect, no UDPase and PEP-phosphatase activities could be detected in the crude

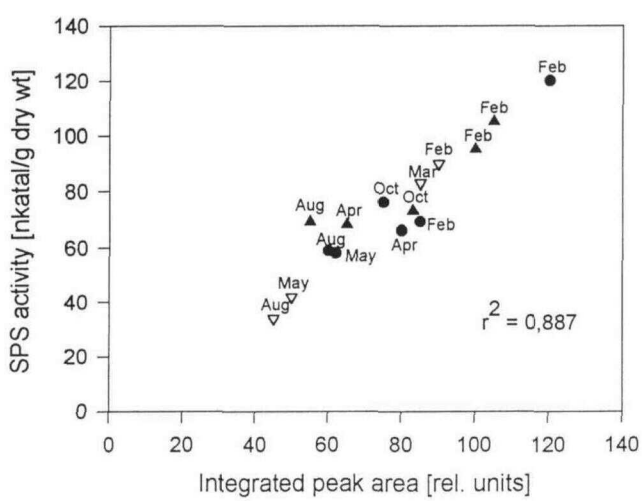

Figure 8. Correlation between SPS activity and SPS protein amount at different times of the year. Integrated peak area representing protein amount was measured by densitometry reading of western blots. Data are values from three individual trees, each indicated by a different symbol.

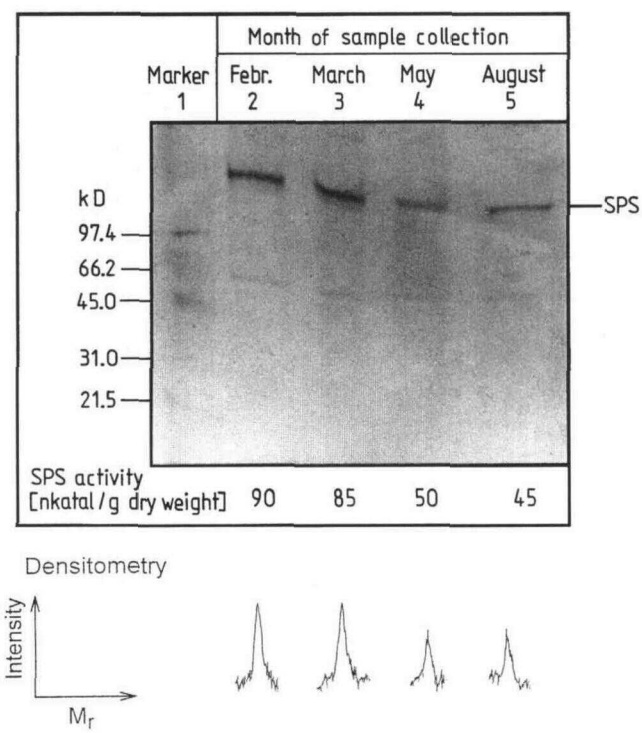

Figure 9. V_{max} activity, immunological detection of spruce needle SPS during the course of a year with a 1:100 dilution of the antiserum raised against spinach leaf SPS, and densitometry reading profile. Lane 1, Protein molecular mass marker; lanes 2 to 5, mature needle samples taken at different times of the year (40 μg dry weight per lane).

extract, and PGI activity was reduced by more than 90% to a tolerable extent by the inhibition of 6-phosphogluconate.

Allosteric regulation of SPS is different among various species (Huber et al., 1989b). Allosteric properties of the spruce enzyme, together with a Glc-6-P-mediated decrease of K_m for Fru-6-P and Pi inhibition only after Glc-6-P activation, are different from all other plants tested so far. In soybean, for example, SPS is not activated by Glc-6-P and only partially inhibited by Pi (Nielsen and Huber, 1989). In contrast, the K_m values for Fru-6-P and UDP-Glc are both affected by Glc-6-P in potato tuber (Reimholz et al., 1994). Maize SPS decreases the K_m for UDP-Glc only after the addition of Glc-6-P (Kalt-Torres et al., 1987), whereas in spinach leaf SPS exhibits a decrease in the K_m for Fru-6-P only (Doehlert and Huber, 1983; Siegl and Stitt, 1990). The inhibitory effect of Pi on spruce needle SPS is similar to that of the maize enzyme, where only the Glc-6-P activation is inhibited by Pi (Kalt-Torres et al., 1987). In contrast, SPS of spinach leaves (Doehlert and Huber, 1983; Siegl and Stitt, 1990) and potato tuber (Reimholz et al., 1994) is inhibited by Pi in the absence of Glc-6-P.

Covalent Modification

V_{max} activity of spruce SPS can be decreased in vitro in the presence of ATP by about 30%. The inhibition of this effect by staurosporine provided evidence for the participation of an endogenous protein kinase, presumably an SPS kinase. The K_m(ATP) for phosphorylation (30 μM) is in the range reported for SPS kinases of other plants, e.g. 50 μM in spinach (Huber et al., 1989a) and 10 μM in maize leaves (Huber and Huber, 1991).

In spinach leaves, there is also a time-dependent decrease in SPS activity after incubation with ATP, but inactivation by 30% occurred only after 60 min under limited Fru-6-P concentrations and in the presence of Pi (Huber et al., 1989a). Maximal activity was not affected, which is in contrast to the properties of the spruce needle enzyme. Incubation of spruce needle SPS with ATP did not alter its allosteric behavior. Consequently, this effect of ATP on spruce needle SPS does not alter affinities for the substrates or effectors but decreases V_{max} activities.

A protein phosphatase activity that could interfere with the kinase reaction was not detected in the crude extracts. This conclusion resulted from the observation that addition of okadaic acid, a phosphatase type 1 and 2A inhibitor, did not alter SPS activity. In addition, the SPS assay was linear for more than 1 h (see also Egger and Hampp, 1993), either in the presence or absence of ATP. Most probably, the concentration of the phosphorylated protein was too low for the native phosphatase in the diluted extract. As discussed above, endogenous metabolites are considered to be present in a concentration too low for them to interact with the kinase reaction.

In summary, a classification of spruce needle SPS into one of the three classes described by Huber et al. (1989b) is not possible because the enzyme exhibits only a slight inhibition by Pi. Thus, a limited activity test cannot be defined. With regard to the absence of a response to dark → light transitions (data not shown), the spruce enzyme exhibits some similarities with the soybean leaf enzyme (Huber et al., 1989b). But in contrast to the spruce needle enzyme, soybean SPS shows only weak allosteric regulation (Nielsen and Huber, 1989). In addition, the properties of spruce SPS, which exhibits strong allosteric Glc-6-P activation, do not seem to be related to covalent modification or light activation, as has been suggested for other plant sources by Nielsen and Huber (1989).

The variation among regulatory properties of SPS from different plants is reflected in the high variability of SPS genes. This can be seen by the alignment of the SPS amino acid sequences of different plant species.

Partial Enzyme Purification

The correlation of the protein level with the SPS activity in mature needle samples strongly indicated that the spinach antibody binds to spruce needle SPS. The calculated mass of approximately 139 kD for the spruce SPS subunit, in addition, is in the same range as reported for potato tuber (124.8 and 133.5 kD; Reimholz et al., 1994), maize (120 kD; Bruneau et al., 1991), and spinach leaf (120 kD; Walker and Huber, 1989). No other prominent proteins of that mass exist in plants, so unspecific binding can be ruled out. The smaller proteins that were detected are presumably products of proteolytic breakdown.

Regulation of Annual Changes

Our data indicate that ATP-dependent phosphorylation does not appear to play a major role in the regulation of seasonal changes of SPS activity. However, such annual changes in spruce needle SPS activity can be correlated with the level of SPS protein. Although no seasonal variation of allosteric control of spruce needle SPS was observed, it still might be an important factor in the regulation of SPS activity in vivo due to seasonal changes in pool sizes of the effective metabolites Fru-6-P and Glc-6-P. In winter, spruce needles exhibit the highest Glc-6-P content, which could lead to a further activation of SPS. The substrate concentration of Fru-6-P, in addition, is also elevated in winter, which is supported by the studies of Einig and Hampp (1990) and Egger et al. (1996). Developmental changes of Fru-6-P in spruce needles were observed only during the early stages of growth, when contents decline and needles achieve capacity for Suc synthesis and carbohydrate export (Hampp et al., 1994). In mature needles (1–5 years old), no further developmental influence on Fru-6-P contents can be found (Einig and Hampp, 1990).

For winter rye (Hurry et al., 1994, 1995a), spinach leaves (Guy et al., 1992), and winter wheat and winter rape (Hurry et al., 1995b), the induction of an increase in SPS activity by low temperatures was described. As in spruce needles, this effect could be due to an increased amount of SPS protein (spinach leaves: Guy et al., 1992; Holaday et al., 1992) or, in contrast to the spruce needle enzyme, to a higher SPS activation state (winter rye, winter rape; Hurry et al., 1994, 1995b). Exposure of overwintering and frost-tolerant plants to low temperatures is paralleled by an accumulation of soluble carbohydrates, e.g. Suc (spinach

leaves: Guy et al., 1992), hexose-Ps (winter rye: Hurry et al., 1994, 1995a), and starch (spinach leaves: Guy et al., 1992; winter wheat and winter rape: Hurry et al., 1995b), as well as an increase in photosynthetic capacity (Holaday et al., 1992; Ölquist et al., 1993; Hurry et al., 1995a). These observations were discussed as being an adaptation to increased freezing tolerance by accumulating Suc and other cryoprotective sugars (Guy et al., 1992; Hurry et al., 1994, 1995a). Furthermore, these metabolic changes are thought to occur to maintain a high photosynthetic capacity, despite increased levels of soluble carbohydrates and phosphorylated intermediates at low temperature (Hurry et al., 1995b). Hurry et al. (1994) suggested that a considerable photosynthetic rate is necessary to maintain metabolism during overwintering.

In spruce needles, the increased SPS activity and the high levels of hexose-Ps indicate a high capacity for Suc synthesis that leads to the accumulation of Suc in winter (Egger and Hampp, 1993). Suc accumulation does not directly inhibit spruce needle SPS activity (data not shown). Suc can be loaded into the phloem for export even in winter under mild conditions (Blechschmidt-Schneider, 1990), which could prevent a long-term accumulation of soluble carbohydrates and phosphorylated intermediates. In addition, Suc is used for the formation of raffinose and stachyose, which are also synthesized during cold adaptation (Kandler et al., 1979; Egger et al., 1996). Because spruce needles produce neither starch (Senser and Beck, 1979; Egger et al., 1996) nor fructans in autumn and winter, the considerable rates of photosynthesis of spruce needles that occur at this time (Pisek et al., 1967; Rinderle, 1990) require an increased capacity for Suc synthesis to recycle Pi. Therefore, as Galtier et al. (1993) deduced from experiments with transgenic tomato plants, we conclude that the increase of SPS activity in cold-adapted spruce needles may be directly linked with photosynthetic activity or a high level of Suc export.

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